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Polymer Production by *Klebsiella pneumoniae* 4-Hydroxyphenylacetic Acid Hydroxylase Genes Cloned in *Escherichia coli*

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The expression of *Klebsiella pneumoniae hpaA* and *hpaH* genes, which code for 4-hydroxyphenylacetic acid hydroxylase in *Escherichia coli* K-12 derivative strains, is associated with the production of a dark brown pigment in the cultures. This pigment has been identified as a polymer which shows several of the characteristics reported for microbial melanins and results from the oxidative activity of 4-hydroxyphenylacetic acid hydroxylase on some dihydroxylated compounds to form *o*-quinones. A dibenzoquinone is formed from the oxidation of different mono- or dihydroxylated aromatic compounds by the enzyme prior to polymerization. We report a hydroxylase activity, other than tyrosinase, that is associated with the synthesis of a bacterial melanin.

Many species of bacteria and fungi have been reported to produce dark brown pigments (5, 23, 27). Among them, the production of melanin has been reported only in *Vibrio* sp. (18, 24) and actinomycetes (35). Melanin is an irregular polymer composed of indoles, benzthiazoles, and amino acids, and its synthesis is dependent upon tyrosinase, a copper-containing enzyme which catalyzes the *ortho*-hydroxylation of monophenols and aromatic amines to *o*-quinones (26). All subsequent steps in melanin biosynthesis (oxidation and polymerization) are nonenzymatic.

We have recently cloned and expressed in *Escherichia coli* the *hpaA* and *hpaH* genes from *Klebsiella pneumoniae* (14). These genes encode a hydroxylase involved in the microbial catabolism of 4-hydroxyphenylacetic acid (4-HPA) (1, 14, 21). When the *K. pneumoniae hpaA* and *hpaH* genes were expressed in *E. coli* strains unable to catabolize 4-HPA, a deep brown pigment appeared in the medium. The purified pigment showed characteristics similar to those of microbial melanins, and analysis of this pigment by gas chromatography-mass spectrometry identified it as a polymer composed of indole and benzene derivative compounds.

This report describes the catalytic activity of 4-HPA hydroxylase from *K. pneumoniae*: the reaction mechanism implies hydroxylation of the substrate, if it is monohydroxylated, and its later oxidation to *o*-quinones. The broad substrate tolerance of this enzyme and the chemical reactivity of quinones indicate that a wide variety of compounds could be utilized as precursors for biosynthesis of differently composed polymers.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. pAG620 contains the *hpaA* and *hpaH* genes and a promoter-operator region on a 3.3-kb *Bam*HI insert (14). Cells were grown on minimal medium (16) containing 5 mM 4-HPA and 20 mM glycerol as a carbon source. L-Tyrosine (L-Tyr) was used at a final concentration of 50 μg/ml. Growth conditions were as described previously (1, 14, 21).

Preparation of cell extracts and protein determination. Bacteria were harvested at the late logarithmic phase of growth and washed with 0.1 M sodium-potassium phosphate buffer (pH 7.5). Crude extracts were obtained as described previously (14, 21).

The protein concentration of extracts was measured by the Bradford method (8) with crystalline bovine serum albumin as the standard.

Enzyme assays. All assays were carried out spectrophotometrically at 30°C in 0.1 M sodium-potassium phosphate buffer (pH 7.5). 4-HPA hydroxylase (EC 1.14.13.-) was assayed and partially purified as described previously (21). Cresolase activity from tyrosinase (EC 1.14.18.1) was measured spectrophotometrically by oxidation of NADH at 340 nm; the method used was that of Horowitz et al. (17). Catecholase activity was assayed spectrophotometrically at 475 nm as described by Horowitz et al. (17). The cells were permeabilized as described by della Cioppa et al. (12).

Characterization of the brown pigment. *E. coli* CC118(pAG6280) cells were grown aerobically in Luria broth medium (LB) (22) until maximal brown pigment production by the culture was achieved. Brown pigment was extracted with ethyl acetate as previously described (3, 5). The brown compound was resuspended in 0.5 ml of ethanol or in 0.5 ml of ethyl acetate, and the solution was used in subsequent analyses. Gas chromatography-mass spectral analysis was performed with a Hewlett-Packard 5890 Series II gas chromatograph equipped with a methyl silicone capillary column (20 m by 0.22 mm [inside diameter]) programmed from 70 to 220°C (4°C/min) and connected to an HP-5971 mass detector. Two-microliter aliquots of the ethyl acetate extracts were injected into the column to identify any experimental intermediates.

Chemical analysis. The absorption spectrum of the brown pigment was determined spectrophotometrically over a range of wavelengths from 190 to 850 nm. Thin-layer chromatography was performed on silica gel plates with a butanol-acetic acid-water mixture (60:30:10, vol/vol) as the solvent. The spots on the plates were visualized by using UV light (302 nm).

Metal analysis by atomic absorption spectrophotometry. The copper content of partially purified 4-HPA hydroxylase was determined by atomic absorption spectrophotometry with a Perkin-Elmer 11009 apparatus with an HGA700 graphite chamber. The protein concentration used was 0.15 mg/ml.

Electron microscopy assays. Samples were prepared as described by Glauert (15) and examined with a JEOL 100B transmission electron microscope.

Chemicals. 4-HPA, 3,4-dihydroxyphenylacetic acid (3,4-DHPA), 3,4-dihydroxybenzoic acid, and L-Tyr were obtained from Sigma. NADH was from Boehringer Mannheim. All of the other compounds used were of the highest purity available.

RESULTS

Influence of medium composition on pigment production. We have recently cloned and expressed in *E. coli* the 4-HPA hydroxylase genes (*hpaA* and *hpaH*) from *K. pneumoniae* in recombinant plasmid pAG620. When this plasmid was present in *E. coli* K-12 strains unable to catabolize 4-HPA and 3,4-DHPA (4), a dark brown color was produced in LB cultures

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Reference
E. coli K-12 strains		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	20
C600	*thr-1 leu-6 thi-1 supE44*	4
E. coli W	Wild type, 4-HPA degrader	14[a]
E. coli W-21	*hpaA recA* (*E. coli* W mutant)	14
K. pneumoniae M5a1	Wild type, 4-HPA degrader	21
K. pneumoniae AG813	*hpaA recA* (*K. pneumoniae* M5a1 mutant)	1
Plasmids		
pUC18	Ap^r; Col E1 origin	20
pAG620	Ap^r; contains *hpaA* and *hpaH* genes from *K. pneumoniae* in pUC18[b]	14
pGA260	Ap^r; contains same insert as pAG620 in opposite orientation	14
pAG622	Ap^r; with 1.1-kb *Bam*HI-*Hin*dIII fragment of pAG620 insert	14
pAG623	Ap^r; *hpaH* gene	14

[a] *E. coli* W was obtained from R. A. Cooper, University of Leicester, Leicester, United Kingdom.
[b] *hpaA* encodes a hydroxylase protein, and *hpaH* encodes a coupling helper protein (unpublished data).

during the stationary phase of bacterial growth. Brown pigment also accumulated in the medium surrounding the bacterial cells when cultures were grown on LB or brain heart infusion plates.

Curiously, the brown pigmentation increased when $CuSO_4$ or $FeCl_3$ was incorporated in the medium, and formation of a brownish to black pigment was also greatly stimulated when $CuSO_4$ plus L-Tyr was added to the LB medium. Similar results were obtained when pGA260 (with the insert in the opposite orientation) was expressed in *E. coli* K-12 strains, such as C600 and CC118. However, when these plasmids were present in cells able to metabolize 4-HPA (*E. coli* W [wild type]), no pigment production was observed. The presence of pAG620 and pGA260 in 4-HPA hydroxylase-defective mutants (*E. coli* W-21 and *K. pneumoniae* AG813) restored the hydroxylase activity (14) but did not result in accumulation of brown pigment on LB and brain heart infusion plates. No pigment production was observed in subclones from pAG620 (plasmids pAG622 and pAG623) which did not encode 4-HPA hydroxylase.

These results indicated that formation of the pigment is mediated by the enzymatic activity of the cloned hydroxylase only when expressed in cells unable to catabolize 3,4-DHPA. Moreover, the increase in brownish pigmentation caused by the presence of both L-Tyr and $FeCl_3$ suggests that the brown pigment is similar to melanin and that the 4-HPA hydroxylase is a tyrosinase-type enzyme.

Characterization of the brown pigment. To establish the chemical and physical properties of the brown pigment, it was isolated and purified from cultures. The absorption spectrum of the supernatant of the brown pigmented produced by *E. coli* CC118(pAG620) cultures (which had a pH of 8), compared with that of noninoculated LB medium, showed peaks at 240.6 and 274.2 nm. When brown-pigmented medium was analyzed and compared with ethanol, a broad peak between 300 and 500 nm was observed. The absorption spectrum of the purified pigment showed two main peaks at 210 and 263 nm in ethanol. The chemical test characteristics of the purified pigment showed similarities to those of melanins of other microorganisms: insolubility in water, solubility in methanol, flocculation of the pigment caused by $FeCl_3$, decolorization by H_2O_2, and solubility in 1 M NaOH (3).

The brown pigment produced by *E. coli* (pAG620 or pGA260) in LB medium was also analyzed by gas cromatography-mass spectroscopy and produced a spectrum (Fig. 1) that corresponds to that of a polymer composed of indole, phenol derivatives, dibenzoquinones, and a structure similar to that of ergotamine (Fig. 1). These results suggest that the brown pigment is a polymer, similar to melanin, formed by catalytic activity of the enzyme expressed by pAG620 or pGA260.

Electron microscopy assays showed the existence of various electron-dense granules produced by *E. coli* CC118 cells expressing the hydroxylase cloned in pAG620 (Fig. 2). These granular particles were not found in bacteria transformed with pUC18, indicating that these electron-dense granules are pro-

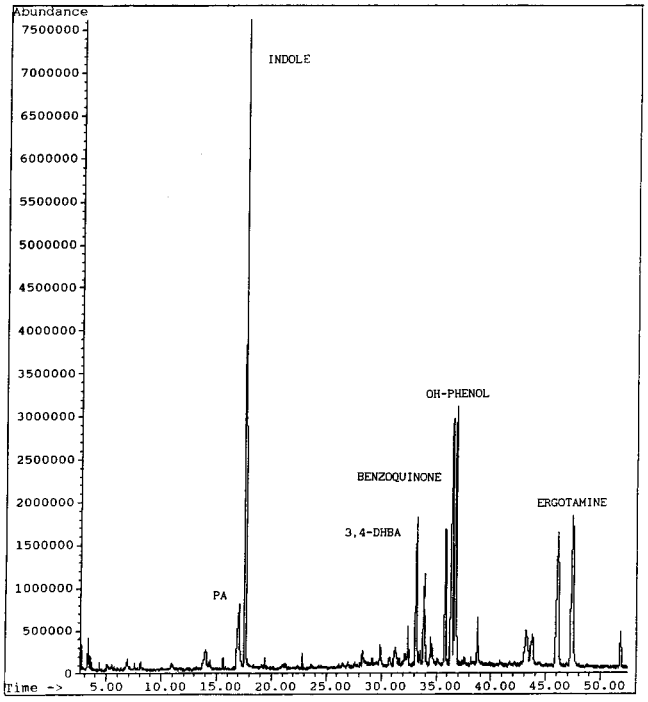

FIG. 1. Chromatographic profile of the brown pigment extracted with ethyl acetate. The peaks of interest were analyzed with an HP-5971 mass detector. PA, phenylacetate.

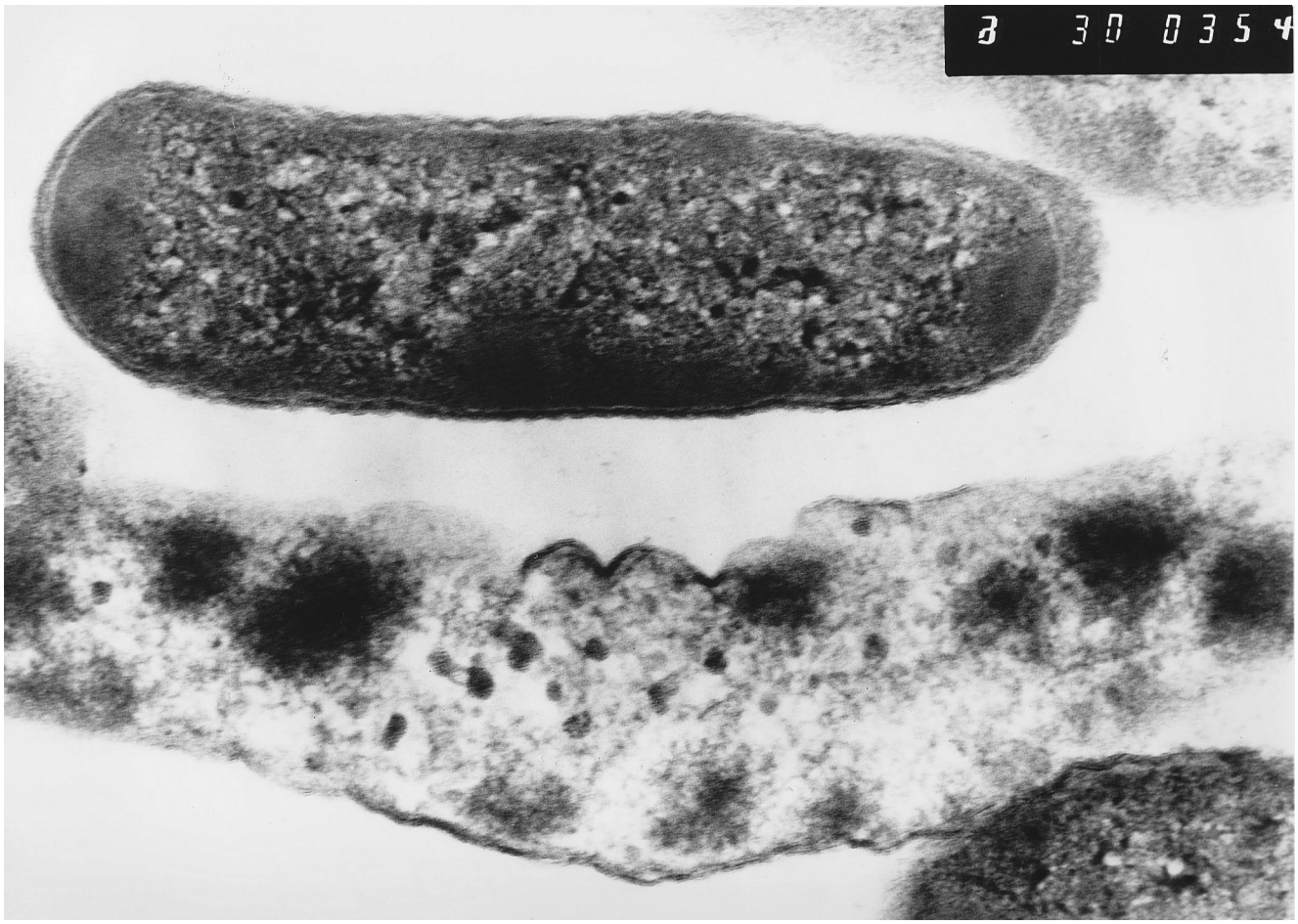

FIG. 2. Electron micrograph of *E. coli* CC118(pAG620) cells. The granular particles located next to the bacterial cell wall were found in 90% of the bacteria transformed with pAG620. None of the cells transformed with pUC18 showed granular particles.

duced by the cloned enzyme. As shown in Fig. 2, the granular particles, with sizes ranging 175 to 400 nm, were located next to bacterial cell walls and were usually elongated. Granular particles related to melanins have been found in many species of bacteria, such as *Aeromonas liquefaciens* (3), *A. salmonicida* (29), *Rhizobium phaseoli* (6), and *Azospirillum brasilense* (27). These observations further suggest that the electron-dense particles found in pAG620-transformed cells (Fig. 2) are heterogeneous polymers similar to melanins.

Substrate specificity. In a previous report (14), we showed that *K. pneumoniae* 4-HPA hydroxylase exhibits a broad substrate specificity range. Furthermore, the enzyme can also oxidize some dihydroxylated aromatic compounds (14), as indicated by analysis of the reaction products formed from 3,4-DHPA and 3,4-dihydroxybenzoic acid (3,4-DHBA) by thin-layer cromatography. Likewise, the absorption spectra of the dihydroxylated compounds showed different values before and after the reaction, and curiously, the absorption spectra of L-3,4-dihydroxyphenylalanine and catechol after the reaction were similar to those of dopaquinone and benzoquinone, respectively (14). These observations suggest that in vitro, 4-HPA hydroxylase catalyzes the enzymatic conversion of the dihydroxylated compounds to quinones. This second enzymatic activity has been confirmed by identification of the products of the reaction by using two different dihydroxylated compounds as substrates, 3,4-DHBA and 3,4-DHPA, which were added to the partially purified hydroxylase. Of particular significance is the fact that both substrates generated similar chromatograms when 2-μl aliquots of the reaction mixtures, extracted 1:1 with ethyl acetate, were injected into the column. The mixture's main component (about 55% as calculated from peak areas) was identified as 1,2-benzenediol, probably arising from enzymatic transformation of 3,4-DHBA or 3,4-DHPA. Another important peak (Fig. 3), amounting to 9.6% of the total, has a mass spectrum characterized by a base peak at $m/z = 225$ and a molecular ion species (35%) at $m/z = 240$. The rest of the fragment peaks were small, the most intense being $m/z = 113$ (10%) and $m/z = 133$ (25%). This small amount of fragmentation suggests an aromatic structure, while the molecular weight is compatible with the elemental composition $C_{14}H_8O_4$ (Fig. 3). Mass spectral features point to a structure similar to that of compound C2, which could also arise from enzymatic transformation involving the condensation of two benzoquinones. After injection of pure samples of the original substrates 3,4-DHBA and 3,4-DHPA under the same analytical conditions, no 1,2-benzenediol or C2 was detected, ruling out the possibility of their origin as chromatographic artifacts or from nonenzymatic transformation.

Although *K. pneumoniae* is not able to grow on L-Tyr, extracts prepared from the cells grown on glycerol–L-Tyr possessed low levels of the enzymes required to catabolize 4-HPA via 3,4-DHPA. Curiously, extracts from *K. pneumoniae* grown

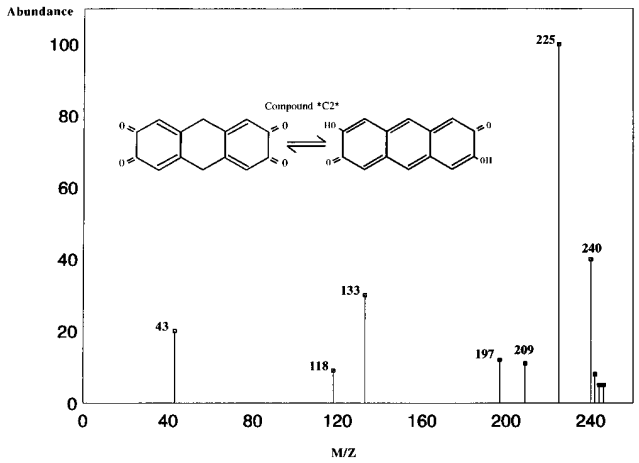

FIG. 3. Mass spectrum of compound C2. This dibenzoquinone is the product of the reaction catalyzed by the cloned hydroxylase with 3,4-DHPA or 3,4-DHBA as the substrate.

on 4-HPA contained tyrosinase activities (Table 2). Likewise, tyrosinase and 4-HPA hydroxylase activities were also measured in cells from *E. coli* CC118(pAG620 or pGA260) grown on LB-ampicillin (Table 2). These results suggest that (i) the hydroxylase is a tyrosinase-type enzyme or that (ii) 4-HPA hydroxylase has the two enzymatic activities described for tyrosinase.

The affinity for substrates (apparent K_ms, 127 μM [4-HPA] and 225 μM [3-HPA]) (21), the complementation of mutants defective in 4-HPA hydroxylase activity, and DNA sequence analysis (GenBank-EMBL data bank accession number L41068) indicate that this enzyme is not a tyrosinase. Moreover, the involvement of copper as the prosthetic group in tyrosinase has been firmly established (19). However, the analysis of 4-HPA hydroxylase by atomic absorption spectrometry showed no copper content in the enzyme. This result led us to conclude that 4-HPA hydroxylase is not a real tyrosinase.

DISCUSSION

We have previously shown that *K. pneumoniae* 4-HPA hydroxylase exhibits a relaxed substrate specificity and in vitro, it also seems to be able to oxidize some dihydroxylated compounds (14). In this work, we studied this second catalytic activity of the hydroxylase.

Expression of the cloned *K. pneumoniae hpaA* and *hpaH* genes in pAG620 in *E. coli* K-12 strains unable to catabolize 3,4-DHPA produced a polymer with structural features similar to those of microbial melanins. Expression of the genes in bacterial cells able to metabolize 3,4-DHPA resulted in no polymer production. Likewise, no brown pigmentation was observed in cells transformed with pAG622 or pAG623, which does not encode the hydroxylase. These data indicate that the formation of this polymer results from the catalytic activity of 4-HPA hydroxylase, which converts an aromatic compound in the medium to a melanin pigment. The low substrate specificity shown by 4-HPA hydroxylase could explain the formation of a dihydroxylated compound which could accumulate in the cells, but how can it explain polymer production from the dihydroxylated compound?

The activity of 4-HPA hydroxylase on dihydroxylated compounds results in its oxidation and subsequent polymerization, as shown by the experiments with 3,4-DHBA and 3,4-DHPA as substrates. In both cases, formation of the intermediate $C_{14}H_8O_4$ shows its capacity to induce the formation of polymers. Apparently, both insertion of molecular oxygen to form the dihydroxylated intermediate and its oxidation to quinone are catalyzed by the same enzyme. Quinones are powerful oxidizing agents and have a tendency to polymerize and condense readily. The formation of quinones as intermediates has also been recently found in bacterial enzymes which, like 4-HPA hydroxylase, are monooxygenases involved in the biodegradation of aromatic compounds (2, 33).

Its melanin pigment production, relaxed substrate recognition, and ability to form quinone from dihydroxylated aromatic compounds suggest that 4-HPA hydroxylase is a tyrosinase. However, the absence of copper bound to this hydroxylase and the substrate affinity for 4-HPA rule out this hypothesis. Interestingly, although tyrosinase has not been explicitly implicated, pigments related to melanins have been found to be produced by many species of bacteria, such as *A. liquefaciens* (3), *A. salmonicida* (29), *R. phaseoli* (6), and *A. brasilense* (27). The biochemical pathways leading to melanin pigment formation in these bacteria are unclear. It has been suggested that brown pigment formation confers protection against toxic chemicals (18). It is possible that in *K. pneumoniae*, 4-HPA hydroxylase is involved in the synthesis of specific compounds that have phenylacetic acids derivatives as structural components. This hypothesis is supported by the fact that some *K. pneumoniae* 4-HPA-negative mutants are also mutants in phenylacetic acid catabolism (unpublished data). Likewise, the extracts prepared from *K. pneumoniae* M5a1 cells grown on glycerol–L-Tyr possessed low levels of the enzymes required to catabolize 4-HPA via 3,4-DHPA. Moreover, 4-HPA is formed as an intermediate

TABLE 2. Brown pigment production associated with 4-HPA hydroxylase and tyrosinase activities in different strains

Strain	Growth substrate	4-HPA hydroxylase activity (mIU · mg of protein)	Tyrosinase activity (mIU · mg of protein)		Brown pigment
			L-Tyr	L-DOPA[a]	
E. coli CC118	LB	<1	<1	<1	No
E. coli CC118(pAG620)	LB-ampicillin	57	6	7	Yes
E. coli CC118(pGA260)	LB-ampicillin	126	12	10	Yes
E. coli W-21(pGA260)	LB-ampicillin	65	7	9	No
K. pneumoniae M5a1	4-HPA	30	5.5	6	No
K. pneumoniae M5a1	Glycyrol–L-Tyr	25	7	8	No[b]
K. pneumoniae M5a1	LB	<1	<1	<1	No
K. pneumoniae AG813(pGA260)	LB-ampicillin	63	6	5	No

[a] L-DOPA, 3,4-dihydroxyphenylalanine.
[b] Formation of a yellow pigment in the culture plates. This yellow pigmentation was associated with accumulation of 5-carboxymethyl-2-hydroxymuconate semialdehyde, an intermediate in 4-HPA metabolism.

during the microbial degradation of phenylacetic acid (7), L-Tyr (30, 32), and phenolic amines such as synephrine (13), tyramine, and octapamine (10, 11) by other bacteria.

In view of the data reported here, 4-HPA hydroxylase is a bacterial enzyme, other than a tyrosinase, that is associated with the production of a melanin pigment. The importance of this process results from the low substrate specificity that this *K. pneumoniae* hydroxylase presents, which enables the synthesis of polymers that differ in composition. The reaction mechanism implies hydroxylation of the substrate, if it is monohydroxylated, and its later oxidation, resulting in compounds with a high capacity to polymerize. This mechanism would be similar to that of the tyrosinase in the synthesis of melanins. Nevertheless, in this process the copper ion in the active tyrosinase plays a relevant role in binding the phenol group of the substrate. 4-HPA hydroxylase does not contain copper in its structure; thus, the substrate binding must involve a different mechanism.

The significance of this work is that it contributes to the knowledge of the mechanism of action of hydroxylases and describes the formation of quinones by 4-HPA hydroxylase. From the industrial application point of view, it could also help establish a protocol for the synthesis of alkaloids and polymers from different precursors, such as amino acids and phenolic compounds, respectively (31). The reaction of the enzyme with certain phenol alcohols (e.g., saligenin) could lead to the formation of a quinone methide which can undergo a number of different reactions to form phenoplasts polymers (9, 25, 28). Otherwise, the formation of a dibenzoquinone from either 3,4-DHPA or 3,4-DHBA and its further condensation to yield compound C2 can result in structures which are often called semiladder polymers (9, 25, 28). These polymers usually have great utility as fibers, films, coatings, and adhesives. Further studies of this enzyme are necessary to explore the biotechnological implications of its enzymatic activity.

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